[CONTRIBUTION FROM THE FRICK CHEMICAL LABORATORY OF PRINCETON UNIVERSITY]

Inhibitors in the Decomposition of Hydrogen Peroxide by Catalase

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In a recent paper Jeu and Alyea[1] found that nineteen different substances have the same inhibitory powers for the autoxidation of sodium sulfite as for the photo-polymerization of vinyl acetate in a hydrogen atmosphere. On the other hand, entirely different inhibitory powers were obtained in studying the photolysis of hydrogen peroxide at 75°. These latter data suggested a useful method for investigating the much-discussed[2] mechanism of the decomposition of hydrogen peroxide by catalase. If the inhibitory powers are the same whether the decomposition is accelerated by catalase or by light, it would be obvious that the reaction chains were being initiated at the surface of the catalase and extending out into the reaction medium in much the same way as the hydrogen–oxygen explosion extends out from the walls of the containing vessel.[3] This work was carried out, then, to decide whether the decomposition occurs at the surface of the catalase or whether the catalase merely initiates chains.

Experimental

Catalase.—Freshest sheep's liver was minced, digested with water and filtered through cheesecloth. The catalase was precipitated from the filtrate by an equal volume of ethyl alcohol, filtered, redissolved in water, and the cycle repeated twice. The final dried precipitate, weighing about 0.5 g., was dissolved in 250 cc. of distilled water, protected from bacteria with a few drops of toluene, and stored in an ice-box. By diluting this solution 30,000 times, a stock catalase solution was prepared which gave measurable rates of decomposition for the hydrogen peroxide. It was stored in ice, and any deactivation of the solution, which amounted to about 10% per month, was made up by the addition of a few drops of the concentrated solution.

Inhibitors.—Merck C. P. products, without further purification, were dissolved in distilled water and stored in Pyrex bottles.

Hydrogen Peroxide.—Merck Superoxol was dissolved in sodium potassium hydrogen phosphate buffer and stored in a Pyrex flask. Decomposition of the peroxide from day to day was compensated for by adding a few drops of Superoxol. The concentration was so adjusted that at the beginning of the run there was 0.05 *M* buffer, P_H 6.80, and 0.05 *M* hydrogen peroxide.

A new Pyrex test-tube was used for each run: 15 cc. of peroxide buffer was run in, followed by a total of 5 cc. of varying ratios of water–inhibitor solutions. The tube was then placed in the bath at 24.0 ± 0.1°, 5 cc. of catalase solution added, the solutions mixed, and a 5-cc. sample immediately titrated with approximately 0.01 *N* potassium permanganate. At five-minute intervals 5-cc. samples were removed and analyzed.

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(1) Jeu and Alyea, THIS JOURNAL, **55**, 575 (1933).

(2) Haldane, *Nature*, **130**, 61 (1932); *Proc. Roy. Soc.* (London), **B108**, 559 (1931); Richter, *Nature*, **129**, 870 (1932); Haber and Willstätter, *Ber.*, **64**, 2844 (1931); Kenner, *Ber.*, **65**, 705 (1932); and others.

(3) Alyea, THIS JOURNAL, **53**, 1324 (1931).

Results

Unfortunately the velocity constants are somewhere between unimolecular and bimolecular. Thus the unimolecular constants of 0.0628, 0.0566, 0.0486 for the first run in Table I are not nearly as satisfactory as the corresponding bimolecular values 0.00315, 0.00322, 0.00303; whereas other uninhibited runs often gave more satisfactory unimolecular than bimolecular constants. To obviate this difficulty, the following device was employed. The dropping values of the unimolecular constants were plotted as in Fig. 1, and the value at the time of mixing ($t = -0.5$) was taken as the true velocity constant. It must be emphasized why this removes the difficulty of deciding which constant to employ. The limiting value of the bimolecular constant $x/a(a - x)$ is x/a^2, while the unimolecular constant $\ln a/a - x$ in the approximate form $(x/a - x) + {}^1/_2(x/a - x)^2$ becomes x/a at vanishingly small values of x. In this paper it is not the absolute velocity constants but rather the *ratios* of these constants which are employed in calculating inhibitory powers. Therefore since a is the same for all runs, the same inhibitory powers will be obtained whether we compare x/a^2 or x/a.

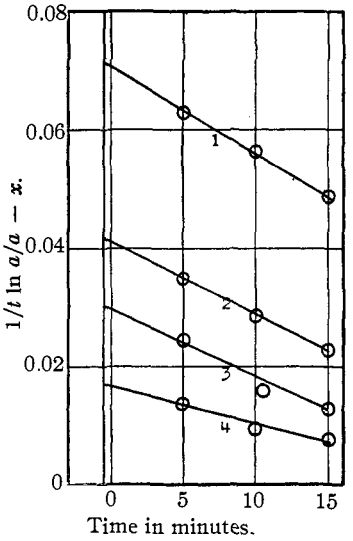

Fig. 1.—Moles peroxide: moles hydroquinone: Curve 1, ∞; Curve 2, 1250; Curve 3, 625; Curve 4, 250.

Catalase Concentration.—Using extrapolated unimolecular constants it was found that the rate of hydrogen peroxide decomposition was proportional to the concentration of the catalase, since peroxide solutions containing 0.1, 1, 5 and 10 cc. of catalase gave constants of 0.0017, 0.0143, 0.0720 and 0.1450, respectively.

Inhibitory Powers.—The effect of inhibitors is expressed satisfactorily by

$$1/t \ln (a/(a - x)) = K/(k_2 + kC) \qquad (1)$$

where a is the initial permanganate titer, $a - x$ the titer at time t, K represents the factor initiating and continuing chains, and $k_2 + kC$ represents the chain breaking by a constant source k_2 and by the added inhibitor of concentration C and inhibitory power k. The significance of this equation is discussed fully in an earlier communication[1] along with the details for calculating the inhibitory powers k, and need not be repeated here. It suffices to state that from the plot of $C/t \ln a/a - x$ against $1/t \ln a/a - x$, the intercepts at the ordinate will give values inversely proportional to

TABLE I

DECOMPOSITION OF HYDROGEN PEROXIDE BY CATALASE INHIBITED BY HYDROQUINONE

Moles peroxide / Moles inhibitor	Time, minutes	Permanganate titer in cc. $(a-x)$	$1/t \ln a/a-x \times 10^4$	Moles peroxide / Moles inhibitor	Time, minutes	Permanganate titer in cc. $(a-x)$	$1/t \ln a/a-x \times 10^4$
∞	−0.5	..	(720)	625	−0.5	..	(300)
	0	23.6	...		0	24.2	...
	5	17.2	628		5	21.4	244
	10	13.4	566		10.5	20.5	158
	15	11.4	486		15	19.9	129
1250	−0.5	..	(417)	250	−0.5	..	(170)
	0	23.6	...		0	24.8	...
	5	19.8	347		5	23.2	136
	10	17.8	285		10	22.6	94
	15	16.8	226		15	22.2	76

the inhibitory power, k. Such a plot has been made for hydroquinone in Fig. 2, using the values in Table I. The values of the curve at the intercepts appear in Table II. Exactly the same was done for the other inhibitors, and the summary of intercepts for all of them is in Table II.

TABLE II

RELATIVE INHIBITORY POWERS

Inhibitor	$1/C$ range	Ordinate $\times 10^4$	Abscissa $\times 10^4$	Corrected ordinate[a] $\times 10^4$	Inhibitory powers (±15%) Catalase 24°	Photolysis 75°
Pyrogallol	625–2500	0.817	829	0.681	3200[b]	3200[b]
Hydroquinone	250–1250	.870	685	.875	2500	8400
Catechol	625–2500	1.38	736	1.24	1750	8200
Resorcinol	125–250	1.54	789	1.35	1600	11000
p-Cresol	250–2500	2.74	626	3.03	720	5600
Phenol	25–250	13.8	690	13.8	160	11000
Propionic acid	2.5–15.5	19.6	711	19.1	110	640
Acetoxime	25–250	23.0	850	18.7	100[c]	1080
Methyl oxalate	25–250	37.8	633	41.6	50[c]	2000
Ethylamine	2.5–25	48.4	690	48.4	45[c]	20000
Benzylamine	6.25–25	62.0	690	62.1	35[c]	12000
Cocaine hydrochloride	25–250	143	656	150	14.5[c]	12000
Chloral hydrate	0.25–2.5	1430	691	1450	1.5[c]	790
Allyl alcohol	10.4–104	...	...	...	<1	150
Benzoic acid	25–250	...	...	...	<1[c]	9800
Benzyl alcohol	5	...	...	...	<1	6700
Ethyl alcohol	2.5–0.25	...	...	...	<1	130
Pyridine	0.25–0.625	...	...	...	<1	1040
Veronal	5–50	...	...	...	<1	1230

[a] Corrected abscissa taken as 0.0690. [b] Taken as standard. [c] May have deactivated the catalase, see Table III.

It is the result of runs at at least three different concentrations of each inhibitor. Simultaneous runs with a given inhibitor all fall along the same

line, as in Fig. 2, but runs made at different times differ slightly due to irreproducibility of catalase activity and other factors. This variation is evidenced by the variation of the intercept at the abscissa in the fourth column of Table II. It is corrected for by taking the abscissa as 0.0690, and using for the ordinate the corrected value of 0.069 uncorrected ordinate/uncorrected abscissa. From the corrected value of the ordinate the relative inhibitory powers can be calculated by taking pyrogallol as the standard (k = 3200) as previously explained.[1] The inhibitory powers, so calculated, are given in the sixth column of Table II, and for reference the seventh column states the inhibitory powers previously found for the photolysis at 75°.

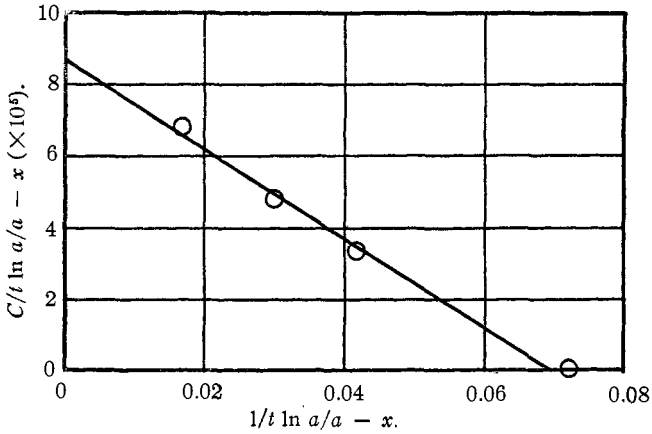

Fig. 2.

Pre-mixing Catalase and Inhibitor.—Another series of experiments was carried out in which 5 cc. of catalase was mixed with 5 cc. of inhibitor

TABLE III

EFFECT OF PRE-MIXING INHIBITOR WITH CATALASE

Inhibitor	Moles peroxide / Moles inhibitor	$1/t \ln a/a - x$ Pre-mixed × 10⁴	$1/t \ln a/a - x$ Not pre-mixed × 10⁴	Inhibitor	Moles peroxide / Moles inhibitor	$1/t \ln a/a - x$ Pre-mixed × 10⁴	$1/t \ln a/a - x$ Not pre-mixed × 10⁴
Allyl alcohol	26	670	690	Acetoxime	25	115	324
Benzyl alcohol	5	650	666		25	70	276
Catechol	250	210	219	Benzoic acid	250	480	690
p-Cresol	250	410	368	Benzylamine	2.5	805	138
Ethyl alcohol	0.25	680	690	Cocaine hydrochloride	25	300	506
Hydroquinone	625	255	294	Chloral hydrate	0.25	0	248
Phenol	62.5	355	345	Ethylamine	6.25	130	200
Propionic acid	6.25	092	099	Methyl oxalate	250	460	666
Pyridine	0.625	715	828				
Pyrogallol	250	92	81				
Resorcinol	125	150	168				
Veronal	5	690	721				

and allowed to stand for twenty minutes at room temperature. At the end of this time 15 cc. of the buffered peroxide solution was added and the run carried through in the usual way. The velocity constants obtained under these conditions are given in Table III. For comparison, constants obtained at the same inhibitor concentration but in which inhibitor, peroxide and catalase were added simultaneously are given in the last column.

Discussion

From Table II it appears that there is no parallelism between inhibitory powers in the reactions accelerated by light and by catalase.[4] The most serious objections to this comparison lie in the fact that the photolysis was carried out at 75° while the catalase reaction is carried out at 24°, and that we know from semi-quantitative experiments on inhibition of the thermal decomposition of hydrogen peroxide that certain of the substances do not inhibit thermolysis and photolysis to the same extent. To correct this, measurements are now in progress at Princeton to compare inhibitory powers for the peroxide decomposition accelerated by various inorganic catalysts as well as by catalase.

It will be noticed that we used buffer in the catalase reaction. The agreement with Equation 1 in which buffer was always present to the same concentration but the inhibitory concentration varied, favors the view that the former did not exert any inhibition. The photolysis experiments were not buffered, the inhibitor concentrations being so low that inhibition was never interpreted as due to change in P_H.

The many separate runs in Table III are not as reproducible as those in Table II, but they afford decisive evidence that catalase is not deactivated by standing at room temperature for twenty minutes with any of the first twelve inhibitors in the table. For if it were, we should anticipate increased inhibition upon standing, and the values in the third column would be lower than those in the fourth column. Doubt exists, however, about the seven inhibitors at the bottom of the table where the rate is slower

(4) This is in disagreement with the conclusions drawn by Schwab, Rosenfeld and Rudolph [*Ber.*, **66**, 661 (1933)] in a recent paper which appeared shortly before the completion of this work. They conclude there is a possible parallelism. Unfortunately they had data for only four inhibitors, two of which did not substantiate parallelism. Ethyl and benzyl alcohols had inhibitory powers of < 0.5 and 1.4, respectively, for decomposition by catalase as contrasted with 130 and 6700 for the photolysis. There are two other corrections to be pointed out. Their second table of inhibitors in various types of reactions consists of six columns of our data and two columns of their own. Their error was in choosing hydroquinone as a standard, particularly in the three columns of sulfite oxidation. For Table X, Jeu and Alyea show that hydroquinone has abnormal inhibition in the sulfite reaction, perhaps due to the formation of a hydroquinone sulfonate. [Alyea and Bäckström, THIS JOURNAL, **51**, 90(1929).] This is why we have always chosen pyrogallol rather than hydroquinone as a standard throughout our work. Second, the use of Equation 1 is urged in calculating inhibitory powers. It is inadvisable to use as a measure of inhibitory powers the reciprocal of the concentration at which equal inhibition occurs, for this holds only when kC is so large that k_2 is negligible, whereby kC becomes inversely proportional to the reaction rate. Now $k_2 = kC$ at 50% reduction in rate. Therefore, under conditions of their paper, which was only 20% reduction in rate, they could calculate only approximate inhibitory powers by this method; whereas with their excellent experimental data very exact inhibitory powers could have been calculated by use of Equation 1.

when the inhibitor is mixed with the catalase twenty minutes before adding the peroxide. Unfortunately these data do not exclude the possibility of the inhibitor deactivating a catalase–peroxide complex, although agreement with Equation 1 rather disparages such a view.

The value of k_2 may be used in calculating minimum chain length according to the method of Jeu and Alyea, to which the reader is referred for details. k_2 is given from the data for pyrogallol in Table II as $3200 \times 0.0000681/0.0690 = 3.16$. The chain length is $6400/k_2 = 2000$. This should be compared with the value of 2000 from the photolysis at 75°.

We wish to thank Mr. J. L. Osmer for preparing the catalase with which this work was carried out.

Summary

1. The decomposition of hydrogen peroxide by catalase has been inhibited by nineteen organic substances.

2. Their inhibitory powers differ markedly from the inhibitory powers previously found for the same substances in the photolysis of hydrogen peroxide at 75°.

3. With twelve of the nineteen inhibitors, allowing a catalase–inhibitor mixture to stand some time before adding peroxide gave the same inhibitory powers as runs in which inhibitor, peroxide and catalase were mixed simultaneously. This is interpreted to mean that the inhibitor did not deactivate the catalase. The remaining seven inhibitors, however, appeared to deactivate the catalase somewhat.

4. The value of minimum chain length, calculated from k_2 in the equation $1/t \ln a/a - x = K/(k_2 + kC)$ according to the method of Jeu and Alyea is 2000.

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